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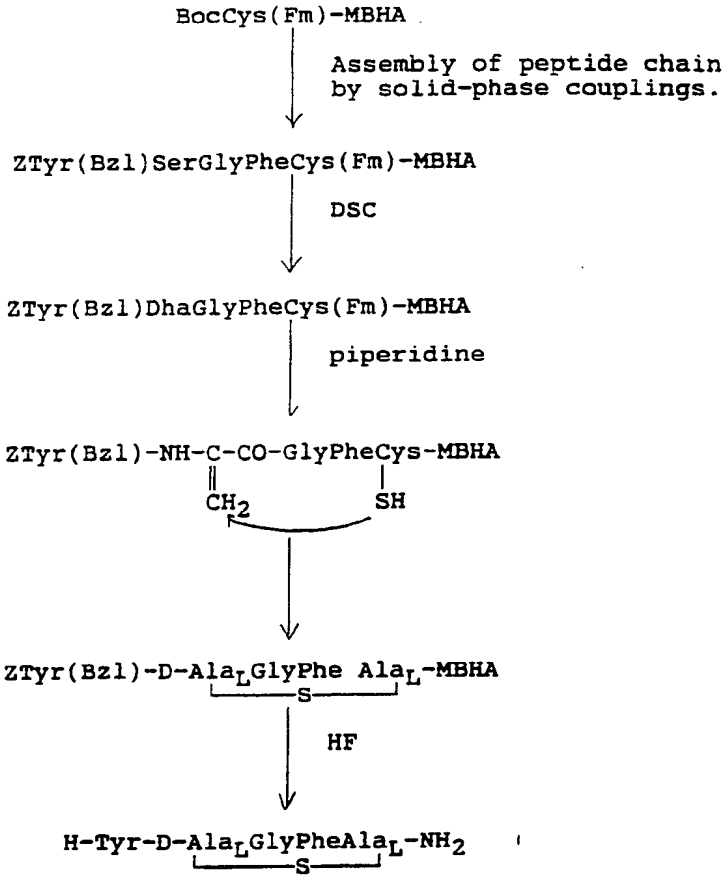
(54) Title: LANTHIONINE BRIDGED PEPTIDES

(57) Abstract

A process for producing peptides having a lanthionine bridge is disclosed. The small cyclic peptides having a thioether bridge are analog compounds of naturally occurring biologically active peptides having improved properties.



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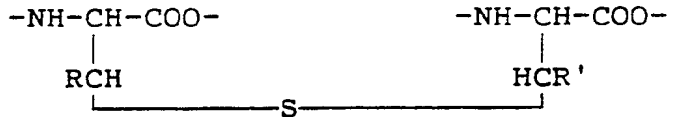
Lanthionine bridged Peptides

It is a basic goal in peptide chemistry to design molecules for medical or industrial application. Design means that naturally occurring peptides which have a biological activity are modified in order to obtain molecules which have advantages over the naturally occurring peptides in different respects. There are several groups of peptides which act as hormones, as neurotoxins or as plant regulating agents. These peptides are usually small, flexible molecules which may optionally have a disulfide bridge.

It is an object of the present invention to provide peptides which comprise a monosulfide bridge. This thioether bond is also designated as a lanthionine bridge and corresponds with the cystine bridge with the exception that the disulfide bridge is replaced by a monosulfide linkage. Two amino acid residues having the general formula



are designated to be joined into a lanthionine bridge wherein the linkage of the two amino acids has the meaning -RCH-S-HCR'-, wherein R and R' respectively represent -H, a lower (C_1-C_{10}) alkyl or aralkyl group. In a preferred embodiment R and R' are H. The amino acid termini of the lanthionine structure are designated as Ala_L if R and R' are

H and Thr_L when R or R' are CH_3. Other ß-substitut d lanthionine components are designated as substituted Ala_L derivatives, e.g. ßethylAla_L.

Thioether bonds of the lanthionine type are known from some fungal toxins and antibiotics, for example from the lantibiotics, as nisin, epidermin, dunamycin or mersacidin. Naturally occurring compounds having the monosulfide bridge always have more than two monosulfide bridges in the molecule.

M.F. Bean et al. have reported in their article "Identification of a Thioether By-product in the Synthesis of a Cyclic Disulfide Peptide by Tandem Mass Spectrometry" as published in the Proceedings of the 11th American Peptide Symposium, ESCOM, (Leiden 1990, p. 443) on a somatostatin analog wherein the internal disulfide bond has been converted to a thioether link. The somatostatin analog with the putative amino acid sequence Phe-Ala-Phe-Trp-Lys-Thr-Ala-Thr(ol), wherein the two Ala_L residues are linked via the thioether bridge, has been described as the by-product which was obtained by the Boc-TFA-preparation of sandostatin analogs. The originally occurring somatostatin derivative has a disulfide bridge.

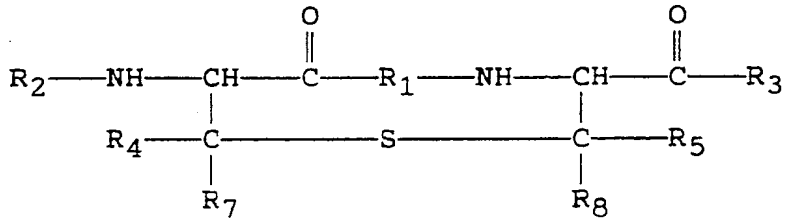
It is an object of the present invention to provide analogs of peptide compounds having at least one monosulfide bridge in the molecule and exhibiting an improved biological activity. The analogs of peptide compounds according to the invention comprise analogs of compounds, such as: ACTH-analogs, angiotensines, magainine, bombesine, bradykinine, fragments of fibronectine, CCK, fragments of hirudine, LHRH-analogs, neuropeptides, neurokinines, neurotensines, substance P, virus related peptides, such as peptides of HIV, thymosine, fragments of thymopoeitin, fragments of oncogenes, atrial natriuric peptides, epidermal growth factors, transforming growth factor and fragments thereof, conotoxines and related

neurotoxines, mast cell degranulating peptides (MCD), urotensine II, HIV gp41 antigenic peptide 1 or peptide 4 or tyrocidin A.

In a preferred embodiment of the present invention the peptide has not more than two monosulfide bridges and in an especially preferred embodiment the peptide has only one monosulfide bridge.

The compounds of the present invention have a higher biological activity than the corresponding naturally occurring peptides.

According to the present invention lanthionine-bridged peptides are disclosed having the general formula



(I)

wherein R_1 is a short sequence of 2 to 10 amino acids selected from the group of the naturally occurring amino acids and the D-enantiomers thereof and

R_2 and R_3 respectively represent naturally occurring amino acids as L- or D-enantiomers or a short sequence of up to 25, preferably 3 amino acids, wherein the N-terminal $-NH_2$ group of the R_2 residue may be replaced by -OH, -H or $-NHCOR_6$ wherein R_6 is an alkyl- or aralkylresidue or the C-terminal -COOH of the R_3 amino acid residue may be replaced by $-CONH_2$ or $-CH_2OH$ or R_2 may represent -H, acyl or aracyl each of them having 1-18 carbon atoms and R_3 may be -OH or $-NH_2$ and $-\overset{O}{\overset{\|}{C}}-R_3$ may be replaced by CH_2OH with the proviso that R_1 is not Phe-Trp-Lys-Thr, when R_2 is Phe and R_3 is Thr(ol), whereby R_1, R_2 or R_3 can also comprise

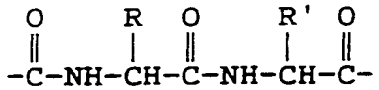
peptidomimetics such as retro-inverso-, carba-, aza-, thiopeptides or peptide rings and wherein R_4, R_5, R_7 and R_8 are hydrogen or an optionally substituted alkyl having 1 to 10 carbon atoms.

In a preferred embodiment of the present invention R_4, R_5, R_7 and R_8 represent hydrogen or a methyl group, wherein each of R_4, R_5, R_7 and R_8 may be a methyl group.

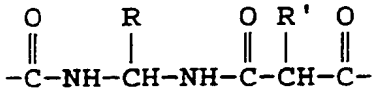
The amino acids can be selected from the group consisting of the naturally occurring amino acids including the L-enantiomers and the D-enantiomers. The group of the naturally occurring amino acids comprises alanine, arginine, asparagine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, ß-alanine, γ-aminobutyric acid, betaine, carnitine, citrulline, creatine, ornithine, saccharopine, 3,4-dihydroxyphenylalanine, 5-hydroxytryptophan, thyroxine, homocysteine, S-methylmethionine, penicillamine, pipecolic acid and nalidixic acid.

The radicals R_1, R_2 or R_3 can also comprise peptidomimetics such as retro-inverso-, carba-, aza-, thiopeptides or peptide rings.

It is understood that a regular peptide has the following structure:



whereas the retro-inverso-structure has the following formula:



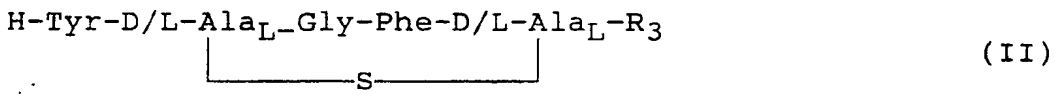
In a preferred embodiment of the present invention the peptides according to the invention of formula (I) have a short sequence of 2 to 7 and most preferably 2 to 4 amino acids representing residue R_1.

Preferably the amino acids of the residues R_2 and R_3 are selected from the group of amino acids comprising D-Phe, D-ß-Nal, Tyr, $TrpNH_2$, $ThrNH_2$, Thr(ol). Alternatively the substituents R_2 can be H, acyl or aracyl with 1 to 18, preferably 2-12 carbon atoms and R_3 can have the

meaning of -OH or $-NH_2$ and $-C-R_3$ can be replaced by CH_2OH. Moreover R_2 and R_3 respectively can be a short amino acid sequence of Pro-Arg-Gly or Pro-Leu-Gly.

In preferred embodiments of the present invention R_1 is represented by a short amino acid sequence selected from the group consisting of Gly-Phe; Phe-D-Trp-Lys-Thr; Phe-D-Trp-Lys-Val; Tyr-Phe-Gln-Asn, Tyr-Ile-Gln-Asn, Tyr-D-Trp-Lys-Val, Gly-Asn-Leu-Ser-Thr, Ser-Asn-Leu-Ser-Thr or Glu-Lys-Asp-Met-Leu-Ser-Ser.

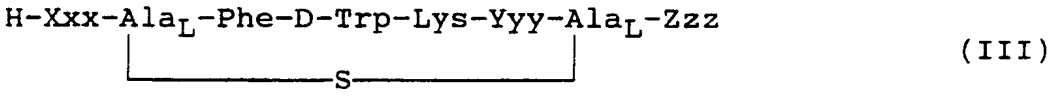
Among the especially preferred peptides of the present invention are: lanthionine-enkephalins having the formula



(II)

wherein R_3 is OH or NH_2,

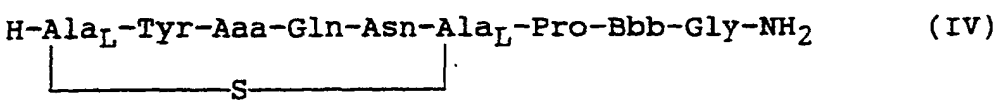
lanthionine-somatostatins having the general formula



(III)

wherein Xxx = D-Phe, D-ß-Nal; Yyy = Thr, Val; Zzz = $TrpNH_2$, $ThrNH_2$ or Thr(ol) with the proviso that Xxx is not Phe when Zzz is Thr(ol) and Yyy is Thr.

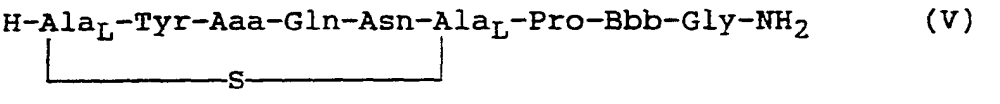
Lanthionine-vasopressin having the formula



(IV)

wherein Aaa = Phe and Bbb = Arg, or

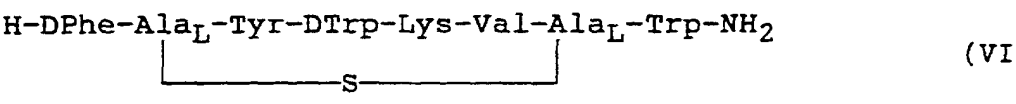
lanthionine-oxytocin



(V)

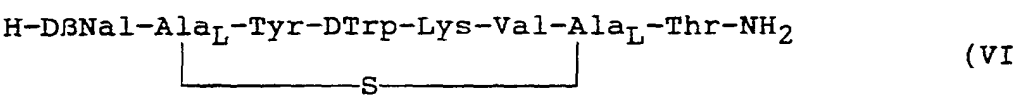
wherein Aaa = Ile and Bbb = Leu.

Further preferred peptides of the present invention have the formula (VI)



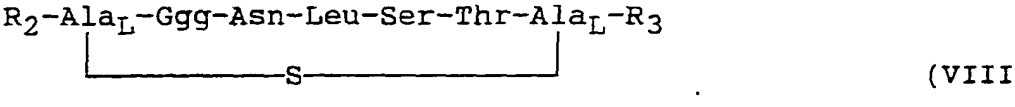
(VI)

or the formula (VII)



(VII)

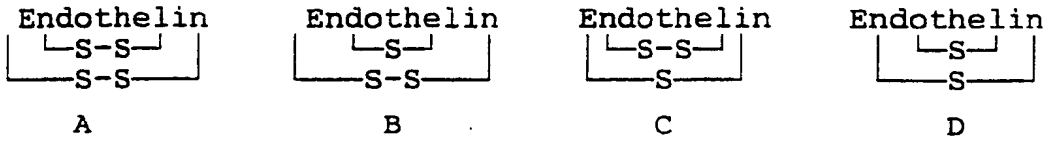
Further preferred peptides have the general formula (VIII)



(VIII)

wherein R_2 is H, acyl or aracyl, R_3 is the fragment 8-32 of human, salmon or eel-calcitonin and Ggg is Gly or Ser.

In another preferred embodiment of the present invention the peptide has the amino acid sequence of endothelin (Schematic Structure A see below) wherein one or two of the naturally occurring disulfide bridges are replaced by a thioether bond. Therefore the compounds can be shown as described by the schematic structures B, C and D.



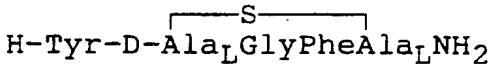
Schematic Structures

The preferred peptides of the present invention will have sequentially overlapping thioether bonds as shown above, if the peptide has two thioether linkages. This means that in the amino acid sequence one lanthionine-bridge is located between two Ala_L residues forming the second lanthionine-bridge.

The peptides of the present invention can be used as pharmaceutically active compounds. They can therefore be used in pharmaceutical compositions comprising at least one of the peptides of the present invention.

Depending on the nature of the biologically active peptide they can be used in injection solutions, capsules, tablets, ointments, creams, sprays and suppositories.

A representative example of the peptides of the present invention can be produced according to the following procedure described for the enkephalin



The linear peptide chain was assembled on a methylbenzhydrylamine resin using *tert*-butoxycarbonyl chemistry with the symmetrical anhydride peptide coupling method. A serine residue was preferably incorporated at position 2 and later on converted to dehydroalanine using disuccinimido carbonate. The S-protecting group (fluorenyl methyl) was selectively removed with piperidine.

A slightly basic milieu, preferably 5% piperidine/dimethylformamide, promoted the Michael addition of the SH-group to the double bond. The amino acid analysis

showed 48% conversion of serine. A greater excess of the reagent disuccinimido carbonate would have resulted in an increased yield for these two steps. "Low-high HF cleavage" was used to cleave the peptide from the resin and to remove the protecting groups. Purification of the resultant crude product was achieved by preparative RP-HPLC using a gradient acetonitrile-water elution. The material obtained was further purified and desalted by gel filtration on a Sephadex G-15 column (20% acetic acid/water).

The product was identified by amino acid analysis and mass spectrometry. Although the Michael addition is not stereoselective, this reaction resulted in only the (2D, 5L) diastereomer. The other diastereomer expected, (2L, 5L) could not be detected in the reaction mixture. Steric hindrance from the solid support near the SH group may be responsible for the stereoselectivity of the addition reaction. The solid phase synthetic approach allows a rapid assembly of lanthionine-bridged cyclopeptides. Figure 1 shows schematically the process according to the invention. The following examples illustrate the present invention.

Abbreviations used in peptide synthesis section

Standard abbreviations for amino acids and protecting groups are followed according to the IUPAC-IUB Joint Commission on Biochemical Nomenclature: *J. Biol. Chem.* **1971**, 247, 977. Abbreviations used: Acm, amidocarboxymethyl; Boc, *ter*-butoxycarbonyl; Bzl, benzyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethane; Dha, dehydroalanine; DMF, dimethylformamide; DIEA, *N,N*-diisopropylethylamine; DSC, disuccinimido carbonate; EtOAc, ethyl acetate; Fm, fluorenylmethyl; Fmoc, fluorenylmethyloxycarbonyl; HOBt 1-hydroxybenztriazole; Ala_L, lanthionine; MBHA, methylbenzhydrylamine resin; Pac, phenylacyl; TFA, trifluoroacetic acid; Tmse, trimethylsilylethyl; Z, benzyloxycarbonyl; NCA, N-carboxyanhydrid; Trt, trityl.

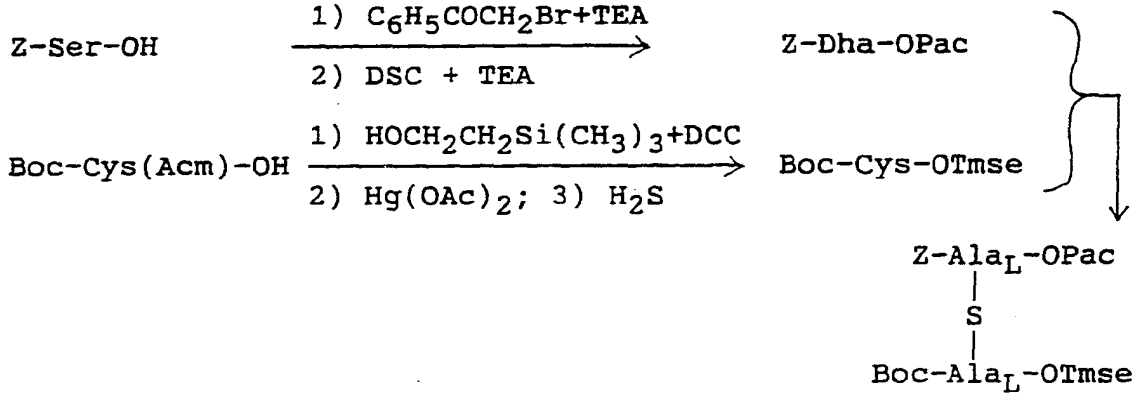
Experimental procedures

All amino acids were of the L-configuration except as indicated. Protected amino acids were purchased from Bachem, Inc. ACS grade solvents (DCM, DMF, acetonitrile) were purchased from Fisher Scientific and purged with nitrogen, then stored over molecular sieves from Sigma. DIEA (Aldrich) was dried over KOH and distilled from ninhydrin. MBHA resin.HCl (Calbiochem) was swollen in DCM and washed with 5% DIEA/DCM followed by DCM before use. TFA, piperidine, DSC (Aldrich) and DCC (Fluka) were used without further purification. Silica gel for flash chromatography was purchased from Baker.

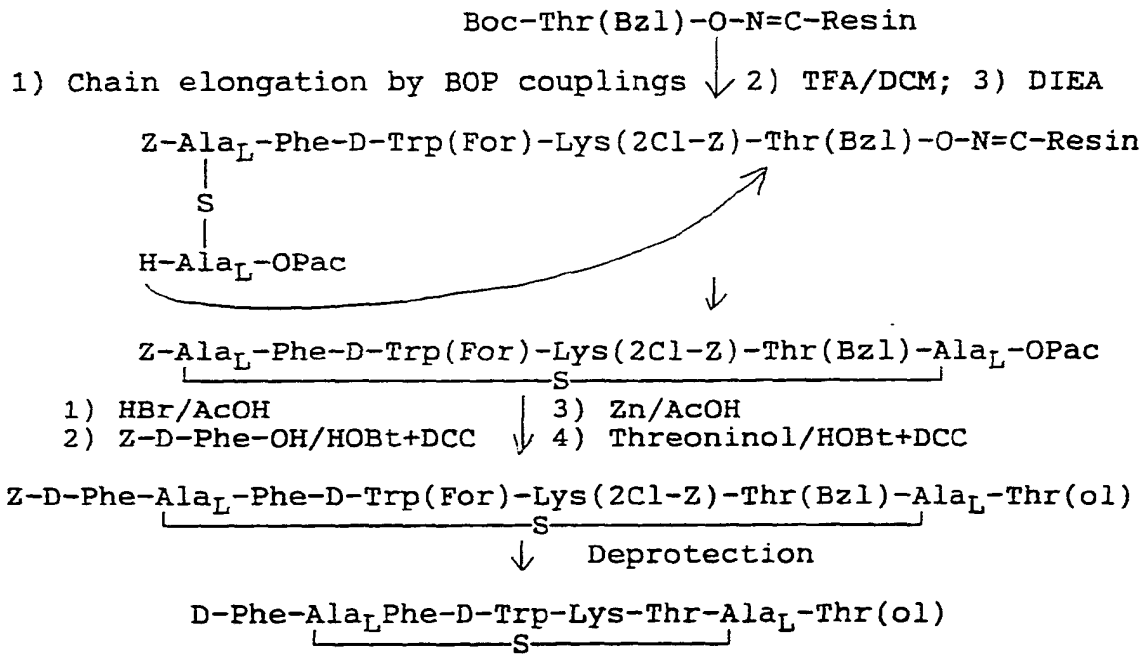
Peptides were analyzed on precoated silica gel 60F-254 plates (Merck) using (A) chloroform:methanol:acetic acid, 65:35:1; (B) butanol:acetic acid:water, 4:1:5 - upper phase. Compounds were visualized by UV, ninhydrin, chlorine/o-tolidine and $KMnO_4$ solution. RP-HPLC analyses were performed on a Waters (Model 510 and Waters 484 detector) instrument with a C-18 analytical column.

There is, however, another synthetic method for the production of peptides. It is often desirable to have diastereomeric peptide analogs. The use of diastereomeric mixture of lanthionine units can provide appropriate diastereomeric analogs, separable by chromatography (HPLC). By such routes, two (or four) analogs can be prepared by a single synthetic process. The simultaneous application of the benzyloxycarbonyl, t-butyloxycarbonyl and phenacyl (or methyl, trimethylsilylethyl, etc.) groups defines the synthetic strategy of this invention to prepare Z-Ala$_L$(Boc-Ala$_L$-OPac)-OH. A new application of the PCOR method (Peptide Cyclization on an Oxime Resin) can provide the new cyclic segment containing a defined lanthionine bridge (Scheme 2), where the chain can be elongated at both termini. The process without a lanthionine bridge has been

described in more detail by Osapay et al. in J. Am. Chem. Soc. 1990, 112, p. 6046-6051 and Tet. Lett. 1990, 31, p. 6121-6124. The final deprotection step followed by chromatographic purification yields the desired compounds.



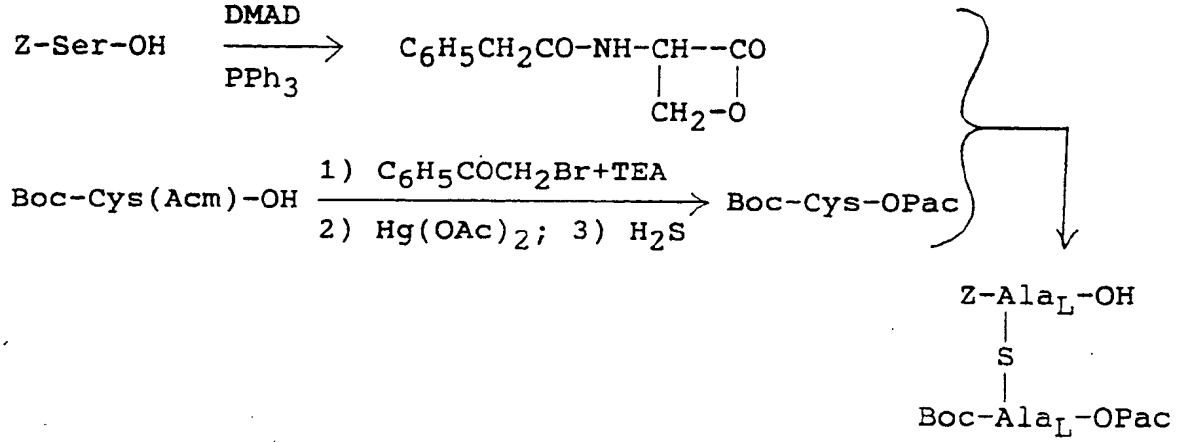
Scheme 1. Synthesis of Protected Lanthionine from Dehydroalanine



Scheme 2. Synthesis of Lanthionine-sandostatin Utilizing Cyclization on an Oxime Resin

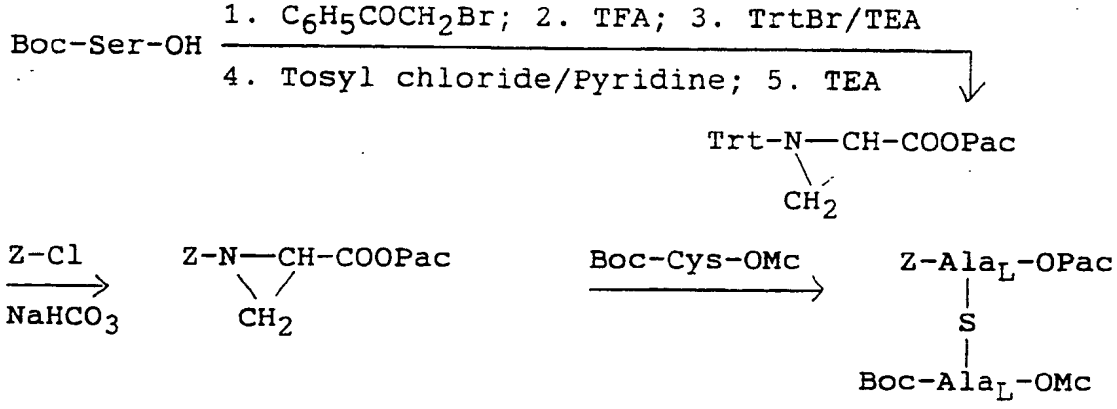
Another highly promising pathway involves the synthesis of two protected intermediates, followed by coupling of the two components in generating an optically pure lanthionine. This is proceeded by the synthesis of both the protected serine

β-lactone (Arnold et al. J. Am. Chem. Soc. 1988, 110, p. 2237-2241) and the protected cysteine. The latter compound acts as a nucleophile in opening the lactone ring at the site of the methylene group (see **Scheme 3**).



Scheme 3. Synthesis of Protected Lanthionine from Serine-lactone

Furthermore another route for the synthesis of the protected lanthionine is disclosed where reactions proceed with retention of configuration. This lanthionine derivative is prepared through the ring opening of an aziridine derivative (Wakamiya et al., Bull. Chem. Soc. Jpn., 1982, 55, 3878-3881) by a nucleophile, namely cysteine or any appropriate SH-containing amino acid (**Scheme 4**).



Scheme 4. Synthesis of Protected Lanthionine from Aziridine Derivatives

Preparation of Lanthionine-opioids

As will be shown later, all of the lanthionine opioids synthesized are superactive both at the μ- and δ -receptor. To investigate structural or pharmacochemical aspects of this new class of opioids, analogs can be synthesized in order to carry out bioassays and conformational analyses of the resulting molecules. Various peptidic or peptidomimetic units can be incorporated into cyclic enkephalin and dermorphin-deltorphin structures including:

Tyr-[D-Ala_L-Gly-Phe-Ala_L]-X, Tyr-[D-Ala_L-Phe-Ala_L]-X and

Tyr-[D-Ala_L-Phe-Asp-Ala_L]-X (X = NH_2 or OH). The incorporation of methyl group(s) at the β -carbon(s) and effects of chirality at the two main chain units of the lanthionine residue can also be included.

Thus the synthesis of β -methyl lanthionines and β,β-dimethyl lanthionine results in modifications, which are expected to lead to substantial differences in bioactivity profiles for closely related target molecules. Thus, critical information about the "bioactive conformations" of the analogs can be obtained. In addition, specific residues such as the Gly in the Tyr-[D-Ala_L-Gly-Phe-Ala_L]-X and the Asp of Tyr-[D-Ala_L-Phe-Asp-Ala_L]-X can be modified with natural and unnatural amino acids. This family of opioids are most promising for obtaining novel and clinically useful opioid drugs.

Lanthionine-somatostatins

New lanthionine-somatostatin derivatives can be synthesized. First, the cyclic segment with a monosulfide bridge of somatostatin or "key-hexapeptide" (**Scheme 2**) or other analogs of somatostatin according to the definition of R_1 on a Kaiser-oxime resin has to be prepared. It will be

elongated at both termini (D-Phe at the N-terminus and threoninol at the C-terminus) to obtain for example the lanthionine analog of Sandostatin. The same synthetic strategy can be used for the preparation of the lanthionine analog of the native somatostatin tetradecapeptide. Potency and receptor selectivity of both target molecules are promising.

D-Phe-Ala_L-Phe-D-Trp-Lys-Thr-Ala_L-Thr(ol)
└──────S──────┘

Lanthionine-sandostatin

AlaGlyAla_LLysAsnPhePheTrpLysThrPheThrSerAla_L
└──────────S──────────┘

Lanthionine-somatostatin

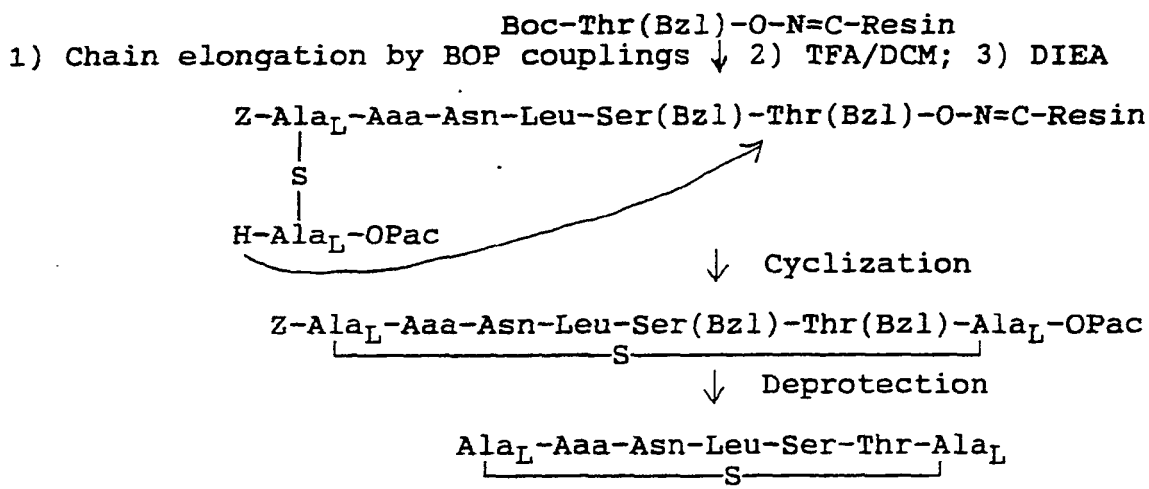
Lanthionine-calcitonins

It is possible to incorporate the lanthionine as the replacement of the cysteine-cysteine disulfide bridge in the N-terminal loop. The loop can be prepared via the PCOR method (**Scheme 5**).

Ala_L-Aaa-Asn-Leu-Ser-Thr-Ala_L
└──────S──────┘

Human and rat calcitonins: Aaa=Gly
Salmon and eel calcitonins: Aaa=Ser

Loops of Lanthionine-calcitonin

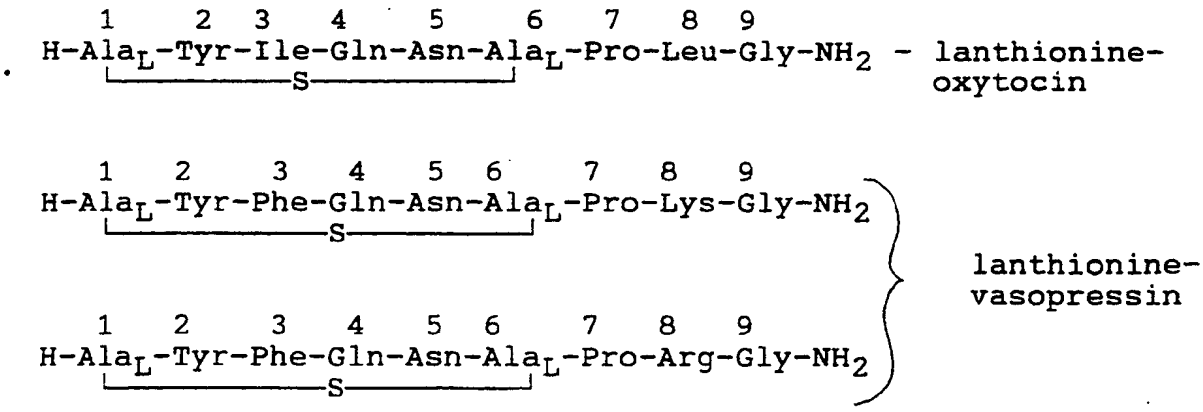


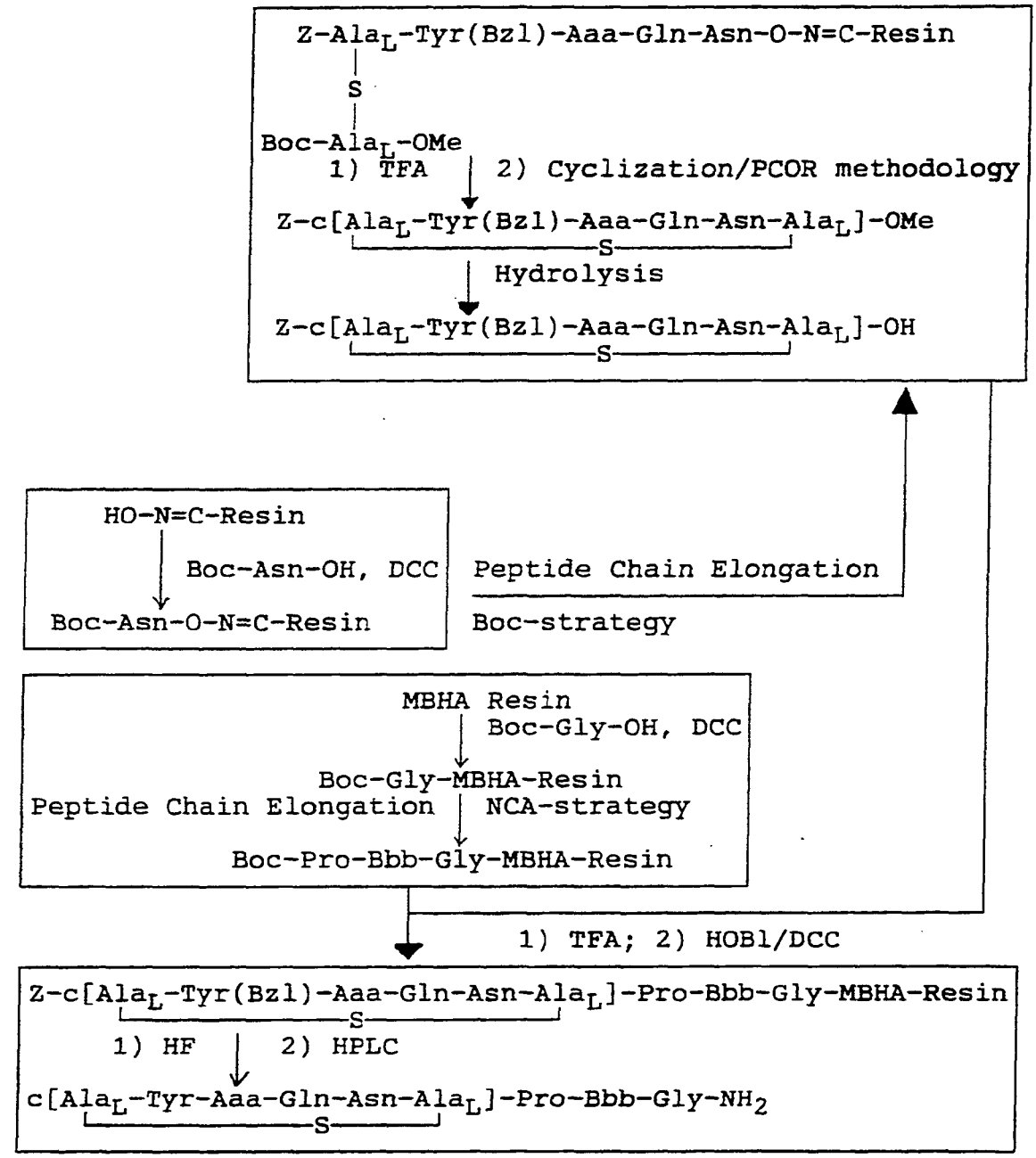
Scheme 5. Synthesis of Loops for Lanthionine-calcitonins Utilizing Cyclization on an Oxime Resin

The elongation at the C-terminus to get the final calcitonin-analog can be performed by normal classical fragment condensations or by the strategy shown later in the paragraph of lanthionine-oxytocin and -vasopressin synthesis (**Scheme 6**).

Lanthionine-oxytocins and Lanthionine-vasopressins

The incorporation of a lanthionine bridge to replace the existing disulfide bridge found in natural oxytocin (OT) and vasopressin (VP) is another example. This can be accomplished by the synthesis of the lanthionine component prior to its incorporation in the peptide sequence (**Scheme 6**).


1 2 3 4 5 6 7 8 9
H-Ala$_L$-Tyr-Ile-Gln-Asn-Ala$_L$-Pro-Leu-Gly-NH$_2$
S
lanthionine-oxytocin
1 2 3 4 5 6 7 8 9
H-Ala$_L$-Tyr-Phe-Gln-Asn-Ala$_L$-Pro-Lys-Gly-NH$_2$
S
1 2 3 4 5 6 7 8 9
H-Ala$_L$-Tyr-Phe-Gln-Asn-Ala$_L$-Pro-Arg-Gly-NH$_2$
S
lanthionine-vasopressin



I Oxytocin: Aaa = Ile; Bbb = Leu

II Lysine-Vasopressin: Aaa = Phe; Bbb = Lys(2Cl-Z)

III Arginine-Vasopressin: Aaa = Phe; Bbb = Arg(NO_2)

Scheme 6. Synthesis of Lanthionine-oxytocins/Lanthionine-vasopressins

Example 1:

a) Preparation of Z-Tyr(Bzl)-Ser-Gly-Phe-Cys(Fm)-MBHA (1)

Methyl benzhydrylamine resin (3 g) was reacted with Boc-Cys(Fm)OH (1.0 g, 2.5 mmol) and DCC (0.52 g, 2.5 mmol) in DCM (30 mL) for 3 hr at room temperature in an SPPS vessel. The remaining amino groups were capped by acetylation. The resulting Boc-Cys(Fm)-MBHA resin (substitution level 0.36 mmol/g, based on picric acid titration) was then deprotected with 30% TFA/DCM (v/v) and neutralized with 1% DIEA/DCM (v/v) solution. The peptide chain was then assembled by consecutive addition of the symmetrical anhydrides (2.5 equiv.) of BocPheOH, BocGlyOH, BocSerOH, and ZTyr(Bzl)OH as well as deprotection steps. The completeness of coupling was monitored by the Kaiser test. Coupling of ZTyr(Bzl)OH was repeated with 1 molar equivalent reagent. Yield 1.06 mmol (84%) peptide based on Gly; amino acid analysis; $Cys_{(1)}Gly_{1.00}Phe_{0.86}Ser_{1.42}Tyr_{1.22}$.

b) Preparation of Z-Tyr(Bzl)-c(D-Ala$_L$-Gly-Phe-Ala$_L$)-MBHA (3)

The protected peptidyl MBHA resin (**1**, 1.06 mmol) in the SPPS vessel was swollen and then suspended in DCM (20 mL). A solution of DSC (387 mg, 1.51 mmol) in acetonitrile (10 mL) was added to the reaction mixture followed by a 5% DIEA/DCM solution (5.22 mL, 1.5 mmol DIEA). The reaction was allowed to proceed for 4 hr, shaking at room temperature in a nitrogen atmosphere. The reaction mixture was drained and the solid phase was washed with DCM (4x). The product (**2**) was treated with a solution of 20% piperidine/DMF solution

(20 mL, v/v, 2x50 min.) and shaken in a 5% piperidine/DMF-DCM solution (40 mL, 1/1, v/v) overnight. The solution phase was drained and the resin was washed with DMF (1x), DCM (2x) and EtOH (2x) and dried. Yield 3.7 g.

c) Preparation of H-Tyr-c(D-Ala$_L$-Gly-Phe-Ala$_L$)-NH$_2$ (4)
S

The peptidyl resin (3, 1.0 g) was treated with anhydrous HF (20 mL) at 0°C in the presence of anisole (1 mL) for 1 hr in a teflon HF apparatus. After removal of volatile components the remaining material was washed with EtOAc (2x20 mL) and the product was extracted with acetic acid followed by 10% acetic acid/water solution. The combined extracts were freeze-dried (yield 200 mg). This material was purified by preparative RP-HPLC on a Vydac C-18 column (1.0 x 25 cm) eluted with 0.1% TFA in acetonitrile/water. A linear gradient from 15% to 22% acetonitrile over 12 min with a flow rate of 10 mL/min was employed. The appropriate fractions were lyophilized to give a solid product (yield 87 mg). Finally, 30 mg of the product was subjected to gel permeation chromatography (1.5 x 100 cm Sephadex G-15 eluted with 20% acetic acid). The peptide fractions were pooled and lyophilized. Yield 16 mg (24% calculated for compound 1). R_F(A) 0.44; R_F(B) 0.49. FAB-MS m/e = 557 (M + 1). Amino acid analysis $Gly_{1.00}Ala_L$-S-$Ala_{L1.1}Phe_{0.99}Tyr_{0.95}$.

Example 2:

Fig. 2 shows schematically the synthesis of lanthionine-enkephalin in solution. Other general methods for chemical synthesis can be followed using mixed anhydrides, carbodiimides, active esters and other coupling procedures. Preferred solvents are CH_2Cl_2 and DMF. Cleavages are

following standard selective reactions. Purification follows well-known extractions, precipitations and chromatographic methods.

Example 3:

The lanthionine-enkephalin was also synthesized by the preferred Fmoc-NCA-Strategy by using the following steps:

(1)	Deprotection (20% piperidine/DMF)	7 min 30 mL/min
(2)	Wash (DMF)	5 min 30 mL/min
(3)	Coupling (see below)	20 min 30 mL/min
(4)	Wash (DMF)	5 min 30 mL/min
(5)	Repeat steps 1-4	

The coupling was performed as follows:
#1: 3 eq. 20 min; #2: 1 eq. + DIEA 20 min

(a) Fmoc-Phe-NCA,
(b) Fmoc-Gly-NCA,
(c) Fmoc-Ser-OH/DCC,
(d) Fmoc-Tyr(Bzl)-NCA.

In the case of peptide chain elongation by the Fmoc-strategy the -SH group of cystein was blocked with a Trt group.

Example 4:

The preparation of lanthionine-bridged cyclic peptide fragments is demonstrated by the following preparations:

a) **Z-D-Ala$_L$-Phe-D-Ala$_L$-OPac**
(S)

Z-D-Ala$_L$-(Boc-D-Ala$_L$-OPac)-Phe-O-oxime resin (1.0 g,
(S)

187 µmol peptide on resin) synthesized by regular solid phase synthetic method was swollen in DCM (10 mL) in a solid-phase peptide synthesis vessel. The Boc group was removed with 25% TFA/DCM, shaking the reaction vessel for 30 min. The peptidyl resin was then drained and washed

(10 mL/wash) with DCM (2x), *i*-PrOH (1x), DCM (2x), *i*-PrOH (1x), and DCM (2x). The amino group was neutralized by treating the peptidyl resin with 5% DIEA in DCM (2 x 1 min.) and then washing with DCM (2x). The cyclization reaction was then carried out by shaking the peptidyl resin in DCM/DMF (1:1, v/v, 10 mL) in the presence of 10 equiv. AcOH at RT for 72 h. The cyclic peptide product was collected from the reaction vessel by draining and then washing the resin with DMF (3x). These solutions were combined and evaporated to a reduced volume, and then washed with water, 0.1 N HCl, 5% $NaHCO_3$, and brine. The solvent was then evaporated and the crude product was purified by silica gel flash chromatography (2 x 20 cm, ethyl acetate-hexanes 1/1). The appropriate fractions were pooled and the solvent was evaporated. The pure solidified product was recrystallized from methanol/ether. Yield 40.5 mg (36.7%); mp 241-244°C (decomp); R_F(EtOAc/hexanes = 2/1) 0.42; FAB-MS m/e = 590 (MH^+); theoretical 590.

b) Z-L-Ala$_L$-Phe-D-Trp(For)-Lys(2Cl-Z)-Thr(Bzl)-L-Ala$_L$-OPac

Z-D-Ala$_L$(Boc-D-Ala$_L$-OPac)-Phe-D-Trp(For)-Lys(2Cl-Z)-Thr(Bzl)-O-oxime resin (100 mg, 6.0 µmol peptide on resin) synthesized by regular solid phase synthetic method was swollen in DCM (1.0 mL) in a solid-phase peptide synthesis vessel. The Boc group was removed with 25% TFA/DCM, shaking the reaction vessel for 30 min. The peptidyl resin was then drained and washed (1.0 mL/wash) with DCM (2x), *i*-PrOH (1x), DCM (2x), *i*-PrOH (1x), and DCM (2x). The amino group was neutralized by treating the peptidyl resin with 2.5% DIEA in DCM (2 x 1 min.) and then washing with DCM (2x). The cyclization reaction was then carried out by shaking the peptidyl resin in DCM/DMF (1:2, v/v, 1.0 mL) in the presence of 10 equiv. AcOH at RT for 72 h. The cyclic peptide product was collected from the reaction vessel by draining and then washing the resin with DMF (3x). These solutions were

combined and evaporated and the crude product was purified by RP-HPLC on a Vydac C-18 column (1.0x25 cm) using 0.1% TFA in acetonitrile water. A linear gradient from 50 to 80% acetonitrile over 15 min., with a flow rate of 4 mL/min., was employed. The product was eluted at 61% acetonitrile and lyophilized to give a solid product. Yield 0.9 mg (24%); R_F($CHCl_3$/MeOH/AcOH = 18/1.5/1) 0.54; FAB-MS m/e = 1,293 (MH^+); theoretical 1,293.

Example 5:

Comparative examples showing the superior biological activity of compounds with the thioether bond compound compared with the compound having the disulfide bridge:

Bioassays Using Isolated Organ Preparations

All of these assays represent standard procedures which have been well described in the literature.

(1) The GPI (guinea pig ileum) assay was performed according to a modified version of a procedure first developed by Paton. Male guinea pigs (300-450 g) were killed by a blow on the skull and exsanguinated. A 2-3 cm segment of ileum not less than 10 cm from the ileocecal junction was mounted in a 20 ml organ bath. The bath contained Krebs' solution of the following composition (in millimolar concentrations): NaCl, 150; KCl, 4.3; $CaCl_2$, 1.25; $MgCl_2$, 1.0; $NaH_2PO_4.H_2O$, 1.7; $NaHCO_3$, 25.0; glucose, 11.0. The temperature was maintained at 37°C and the solution was bubbled with 95% O_2/% CO_2. A GRASS E 2B electrode was used as anode with the 1.5 cm platinum wire entirely enclosed within the lumen. The other end of the preparation was tied over a piece of stiff polyethylene tubing (4 cm, 2.5 mm O.D) which

projects out of the bath solution and was tied to the strain gauge. Another GRASS E 2B electrode was placed about 5 mm from the intestine and parallel to it to achieve transmural stimulation. Single pulses of 4 msec during were delivered by a Harvard apparatus stimulator at a frequency of 10 min^{-1}. Voltages in the range from 3 to 6 V were applied in order to obtain maximal response. Isometric contractions of the ileum were recorded via a Harvard isometric force transducer on a Harvard apparatus biograph which has been calibrated to produce a pen displacement of 1 cm per tension change of 1 g. The results were standardized by expressing the reduction in tension obtained at each dose level as a percentage of the mean tension produced by at least ten preceding control stimulations. Semilogarithmic plots of percent inhibition as a function of peptide concentration permit the determination of IC50-values which were taken as the intercept of 50% inhibition.

(2) The MVD (mouse vas deference) assay was performed essentially as described by Henderson. Briefly, adult, male albino mice (Swiss Webster 30-50 g) are killed by cervical dislocation and the vas deferentia are dissected out. After removal of extraneous fat and connective tissue, the vas is stripped of its associated blood vessel and the somen is gently expressed from the lumen. The vas is then mounted under 0.5 g tension in a 5 ml organ bath containing warmed (37°C), oxygenated (95% O_2, 5% CO_2%) , Mg^{2+}-free Krebs solutions of the following composition [mM]: NaCl, 118; $CaCl_2$, 2.54; KCl, 4.75; KH_2PO_4, 1.19; $NaHCO_3$, 25; glucose, 11; L-tyrosine, 0.2. A modified Harvard apparatus stimulator is used to deliver repetitive field stimulation through platinum wire ring electrodes at the top and bottom of the bath, consisting of twin, rectangular pulses (80 V, 0.15 Hz, 10-ms delay, 1.0-ms duration). Contractions of the muscle are recorded via a Hewlett-Packard Model FTA-1-1 force

transducer connected to a Hewlett-Packard 7702B recorder. Determination of the reduction in the twitch height at various doses permits the construction of log dose-response curves and the determination of IC50-values.

GPI and MVD Assays of Enkephalin Analogs

Compounds	GPI IC_{50} [nM]	GPI Rel. potency	MVD IC_{50} [nM]	MVD Rel. potency
H-Tyr-D-Ala_LGlyPheAla_L-NH_2 (S)	0.62	396	0.54	21
H-Tyr-D-CysGlyPheCys-NH_2 (S—S)	1.51	132	0.76	17
Leu^5-enkephalin	246	1	11.4	1

Table 1

Table 1 shows that the compound according to the invention which has a thioether bond is in both methods, particularly in the most relevant GPI test more potent than the corresponding -S-S- compound.

Example 6:

Using protocols described by Schiller et al., Biochem. Biophys. Res. Commun. 1983, 115, p. 864-870, we compared the half-lives of three compounds: Leu^5-enkephalin, disulfide-enkephalin, and lanthionine-enkephalin. As indicated in **Table 2**, the lanthionine-enkephalin is much more stable than the other two compounds.

Table 2. Enzymatic Degradation of Enkephalin Analogs

Analog	$t_{1/2}$ (min)
Lanthionine-enkephalin	1223
Disulfide-enkephalin	332
Leu^5-enkephalin	30

Example 7:

The lanthionine opioid is highly active in the *in vitro* and *in vivo* tests (**Table 3**). *In vivo* bioactivity was determined using the rat hot plate test after intrathecal dosages.

Tyr-c[D-Ala_L-Gly-Phe-Ala_L]-NH_2 shows 37 times higher bioactivity than morphine and twice the activity of DCLCE (**Table 3**). In the same tests, [Leu^5]-enkephalin shows only 40-50% of full agonistic activity after 100 µg dosage in the *in vitro* assays using GPI and MVD preparations. The lanthionine opioid exhibits 400 times greater bioactivity at the GPI (µ-receptor) and 20 times greater bioactivity at the MVD (δ -receptor) than [Leu^5]-enkephalin. These values are higher than those of its disulfide bridged counterpart, DCDCE. The lanthionine analog does not show a preference for the µ- or the δ -receptor. The IC_{50} ratio (MVD/GPI) is 0.9.

Table 3. Biological Activities of the Lanthionine Bridged Enkephalin Analogs

Analog	GPI[a] IC_{50} [nM]	MVD[a] IC_{50} [nM]	MVD/GPI IC_{50}-ratio	*In Vivo*[b] ED_{50} [nmol]
Tyr-c[D-Ala_L-Gly-Phe-Ala_L]-NH_2 (S–S)	0.62	0.54	0.9	0.11
Tyr-c[D-Cys-Gly-Phe-Cys]-NH_2	1.51	0.76	0.5	0.24
Leu^5-Enkephalin	246	11.4	0.05	>100[c]
Morphine	58.6[d]	644[d]	11	15

a: The GPI and MVD activities are measured in Dr P.W. Schiller's laboratories.

b: Values are measured in Dr T. Yaksh's laboratories.

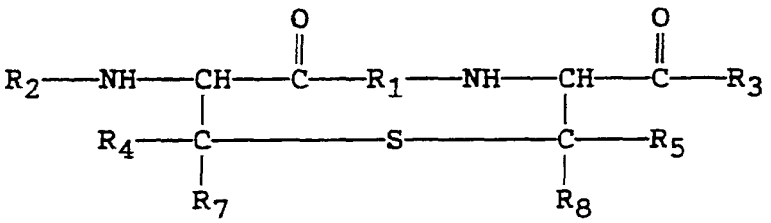
c: The molecule shows 50% of maximum effect at 100µg dosage.

d: Salvadori et al. Hoppe-Seyler, Z. Physiol. Chem. 365:1199, 1984.

Although the lanthionine-enkephalins according to the invention possess superactivity, they do not seem to have high receptor selectivity. To improve their selectivity, it is possible to introduce one or more alkyl (methyl) group(s) in β-position(s) of the lanthionine segment.
In this case at least one of R_4, R_5, R_7, R_8 may be alkyl (methyl).

C L A I M S

1) Lanthionine-bridged peptides with the general formula



(I)

wherein R_1 is a short sequence of 2 to 10 amino acids selected from the group of the naturally occurring amino acids and the D-enantiomers thereof and

R_2 and R_3 respectively represent naturally occurring amino acids as L- or D-enantiomers or a short sequence of up to 25, preferably 3 amino acids, wherein the N-terminal $-NH_2$ group of the R_2 residue may be replaced by -OH, -H or $-NHCOR_6$ wherein R_6 is an alkyl- or aralkylresidue or the C-terminal -COOH of the R_3 amino acid residue may be replaced by $-CONH_2$ or $-CH_2OH$ or R_2 may represent -H, acyl or aracyl having 1-18 carbon atoms and R_3 may be OH or NH_2 and $\overset{O}{\overset{\|}{C}}R_3$ may be replaced by CH_2OH with the proviso that R_1 is not Phe-Trp-Lys-Thr, when R_2 is Phe and R_3 is Thr(ol), whereby R_1, R_2 or R_3 can also comprise peptidomimetics such as retro-inverso-, carba-, aza-, thiopeptides or peptide rings and wherein R_4, R_5, R_7 and R_8 are hydrogen or an optionally substituted alkyl having 1 to 10 carbon atoms.

2) Peptides according to claim 1, wherein R_1 is a short sequence of 2 to 7, especially 2 to 4, amino acids.

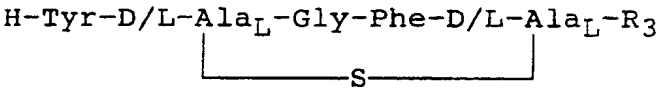
3) Peptides according to claim 1 or 2, wherein R_2 and R_3 represent one amino acid selected independently from each other from the group consisting of D-Phe, D-ß-Nal, Tyr,

$TrpNH_2$, $ThrNH_2$, Thr(ol) or represent the amino acid sequence of Pro-Arg-Gly or Pro-Leu-Gly or wherein R_2 is -H, acyl or

aracyl and R_3 is -OH or $-NH_2$ and the group $-C-R_3$ can be replaced by CH_2OH.

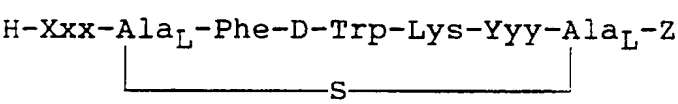
4) Peptides according to claim 3, wherein R_1 represents an amino acid sequence selected from the group consisting of Gly-Phe, Phe-D-Trp-Lys-Val, Phe-D-Trp-Lys-Thr, Tyr-D-Trp-Lys-Val, Tyr-Phe-Gln-Asn, Tyr-Ile-Gln-Asn, Gly-Asn-Leu-Ser-Thr, Ser-Asn-Leu-Ser-Thr or Glu-Lys-Asp-Met-Leu-Ser-Ser.

5) Peptides according to claim 4 having the general formula



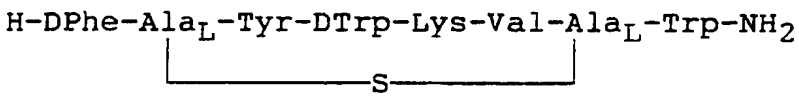
wherein R_3 is OH or NH_2.

6) Peptides according to claim 4 having the general formula

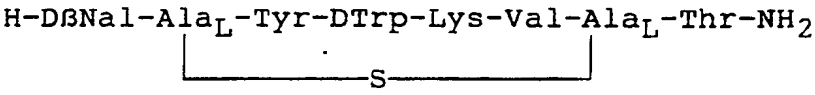


wherein Xxx represents D-Phe, D-ß-Nal; Yyy is Thr or Val, and Zzz is $TrpNH_2$, $ThrNH_2$ or Thr(ol) with the proviso that Xxx is not D-Phe if Zzz is Thr(ol).

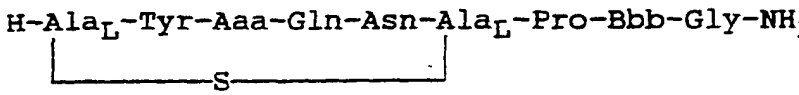
7) Peptides according to claim 4 having the general formula



or

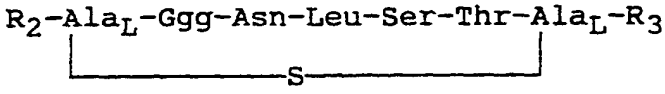


8) Peptides according to claim 4 having the general formula



wherein Aaa is Phe or Ile and Bbb is Arg or Leu.

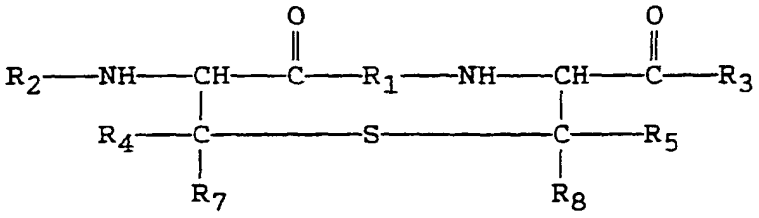
9) Peptides according to claim 4 having the general formula



wherein Ggg is Gly or Ser, R_2 is -H, acyl or aracyl each having 1-18 carbon atoms and R_3 represent the fragment 8-32 of human, salmon or eel-calcitonin.

10) Peptides according to claim 1 having the amino acid sequence of endothelin or related peptides wherein one or two of the two disulfide bridges are replaced by one or two thioether bonds and where the rings are sequentially overlapping.

11) Pharmaceutical composition characterized in that it contains an effective amount of at least one peptide of the general formula



(I)

wherein R_1 is a short sequence of 2 to 10 amino acids selected from the group of the naturally occurring amino acids and the D-enantiomers thereof and

R_2 and R_3 respectively represent naturally occurring amino acids as L- or D-enantiomers or a short sequence of up to 25, preferably three amino acids, wherein the N-terminal

$-NH_2$ group of the R_2 residue may be replaced by -OH, -H or $-NHCOR_6$ wherein R_6 is an alkyl- or aralkylresidue or the C-terminal -COOH may be replaced by $-CONH_2$ or $-CH_2OH$ or wherein R_2 represents -H, acyl or aracyl and R_3 may be -OH or

$-NH_2$ and $-CR_3$ may be replaced by CH_2OH whereby R_1, R_2 or R_3 can also comprise peptidomimetics such as retro-inverso-, carba-, aza-, thiopeptides or peptide rings and R_4, R_5, R_7 and R_8 represent hydrogen or an optionally substituted alkyl having 1 to 10 carbon atoms.

12) Pharmaceutical composition characterized in that it contains an effective amount of at least one peptide according to any one of claims 2 to 10.

13) Process for the preparation of a peptide as claimed in any one of claims 1 to 10 using an appropriate combination of solid-phase peptide synthesis and/or classical synthesis, wherein at least one of the peptide fragments contains a moiety which is cyclized either attached to the resin used or after cleavage from the resin to the desired lanthionine-bridged cyclic peptide fragment which can optionally be elongated at the N- and/or C-terminal to form the final peptide by fragment condensation or step by step synthesis.

14) Process for the preparation of a peptide as claimed in claim 13 characterized in that

- the peptide fragments containing the moiety to be cyclized is assembled on an appropriate resin using *tert*-butoxycarbonyl-chemistry with any peptide coupling method,

- serine is incorporated at the desired place, which is then converted to dehydroalanine using disuccinimido carbonate,

- the S-protecting group attached to the cysteine coupled at the desired place is selectively removed,

- the Michael addition of the SH group to the double bound is promoted by a slightly basic milieu and

- the peptide and the other protecting groups are cleaved from the resin by treatment with HF.

15) Process according to claim 14 characterized by assembling the peptide chain at any appropriate resin using the Fmoc-strategy with any usable coupling agent, intermediately using cleavage of the Fmoc-protecting-group by the piperidine-method, wherein the cleavage of the acid labile S-protecting-group is carried out by any appropriate acid or reagent.

16) Process of preparation of a peptide as claimed in any one of the claims 13 to 15, using the classical synthesis in solution as fragment-condensation or as a step by step synthesis, wherein any known coupling method of appropriately protected amino acids and any cleavage-method of fragments and the final peptide is used.

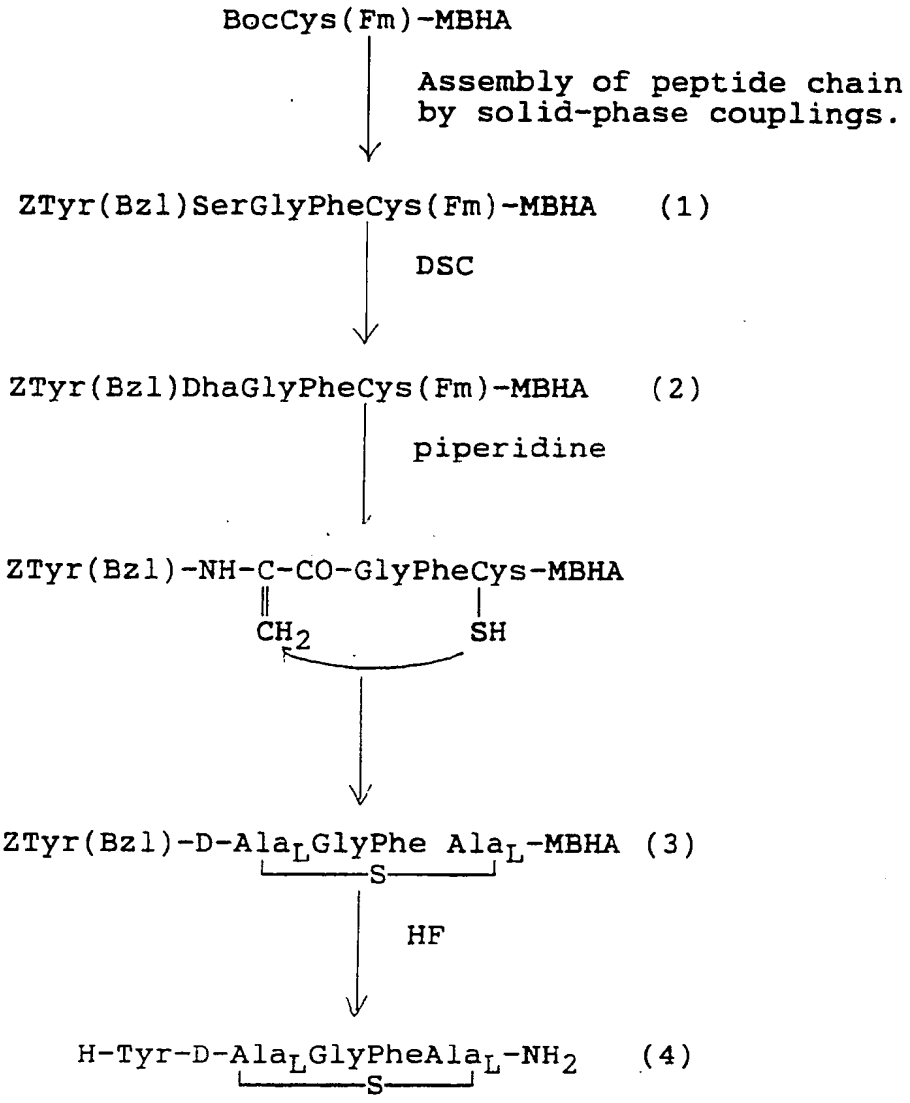
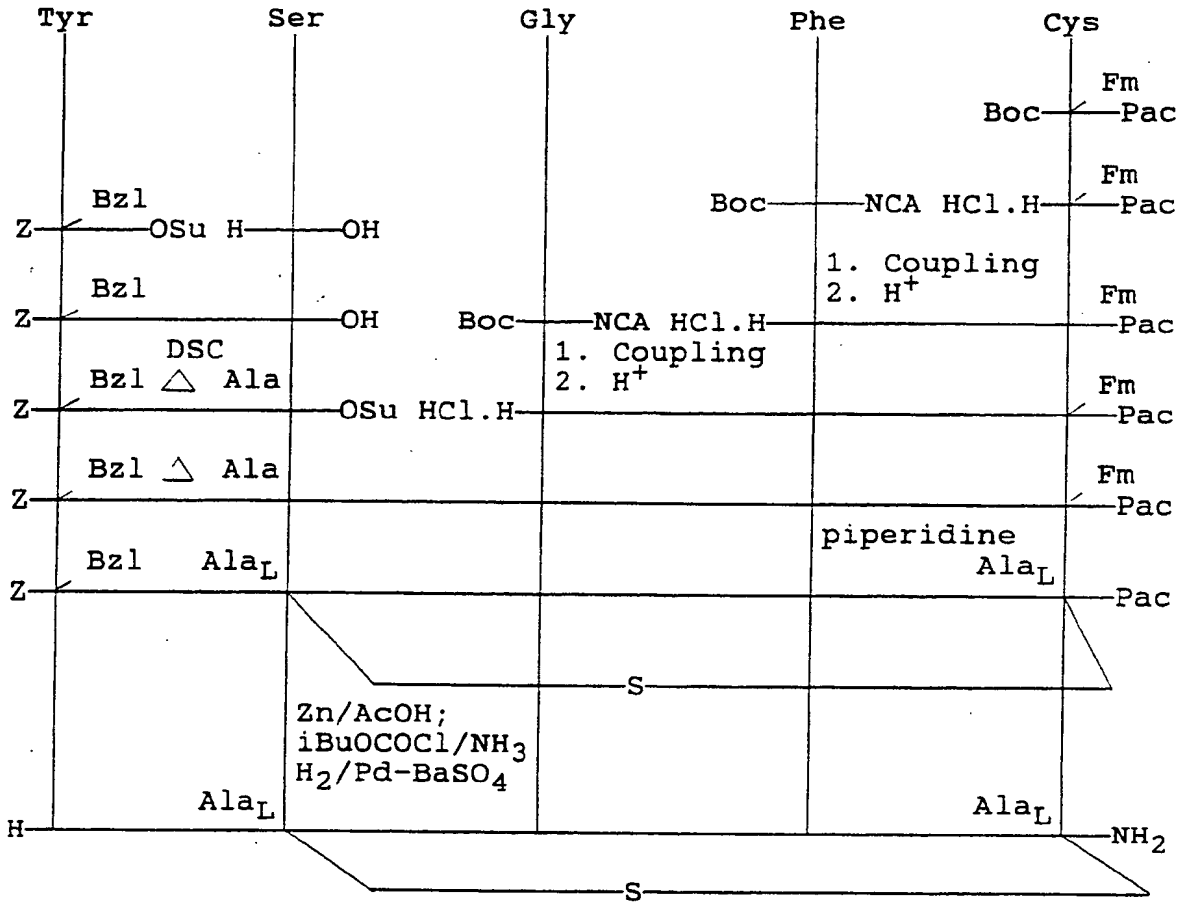

BocCys(Fm)-MBHA
Assembly of peptide chain by solid-phase couplings.
ZTyr(Bzl)SerGlyPheCys(Fm)-MBHA (1)
DSC
ZTyr(Bzl)DhaGlyPheCys(Fm)-MBHA (2)
piperidine
ZTyr(Bzl)-NH-C-CO-GlyPheCys-MBHA
CH2
SH
ZTyr(Bzl)-D-AlaLGlyPhe AlaL-MBHA (3)
S
HF
H-Tyr-D-AlaLGlyPheAlaL-NH2 (4)
S

Fig. 1

Fig. 2

SYNTHESIS OF LANTHIONINE-ENKEPHALIN IN SOLUTION



INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 92/01789

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K7/06; C07K7/08; C07K7/10; C07K7/16
C07K1/04; A61K37/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	EP,A,0 113 029 (MERCK & CO.) 11 July 1984 see examples 5,7 ---	1,6-7, 11-13
A	FR,A,2 320 109 (MERCK & CO.) 4 March 1977 see claim 2 ---	1,5-6, 11-13
A	PEPTIDES. CHEMISTRY, STRUCTURE AND BIOLOGY. PROCEEDINGS OF THE XI AMERICAN PEPTIDE SYMPOSIUM, JULY 9-14, LA JOLLA, CA, USA. 1990, ESCOM, LEIDEN pages 443 - 445 M F BEAN ET AL. 'identification of a thioether by-product in the synthesis of a acyclic disulfide peptide by tandem mass spectrometry' cited in the application see the whole document ---	1-16

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 NOVEMBER 1992	21. 12. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	p. Pasturzo

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	TETRAHEDRON LETTERS. vol. 25, no. 20, 1984, OXFORD GB pages 2067 - 2068 M LEBL ET AL 'synthesis of cyclic peptides by solid phase methodology' see the whole document	1,4,9-16
A	COLLECTION OF CZECHOSLOVAK CHEMICAL COMMUNICATIONS. vol. 39, no. 10, October 1974, PRAGUE CS pages 2835 - 2856 K JOST ET AL. 'synthesis and some biological activities of analogues of deamino-vasopressin with the disulphide bridge altered to a thioether bridge' see the whole document	1,4,9-16
A	PEPTIDES. CHEMISTRY, STRUCTURE AND BIOLOGY. PROCEEDINGS OF THE XI AMERICAN PEPTIDE SYMPOSIUM, JULY 9-14, LA JOLLA, CALIFORNIA, USA 1990, ESCOM, LEIDEN pages 865 - 869 G JUNG 'peptides with sulfide bridges and dehydroamino acids: their prepropeptides and possibilities for bioengineering' cited in the application see the whole document	1,13-16
P,X	JOURNAL OF THE AMERICAN CHEMICAL SOCIETY. vol. 114, no. 14, 1 July 1992, GASTON, PA US pages 5634 - 5642 D E PALMER ET AL. 'effects of dehydroalanine on peptide conformation' see the whole document	1-16

INTERNATIONAL SEARCH REPORT

ational application No.
PCT/EP 92/01789

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see annex

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

The virtually infinite list of the peptides where a disulphide bridge may be substituted with a lanthionine bridge makes a complete search impossible. Therefore, the search was limited to the real examples given in the application, as defined through the claims 4-10, especially referring to the individual peptides endothelin, somatostatin, oxytocin, vasopressins and calcitonin and their analogues and homologues

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9201789 SA 63094

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 13/11/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0113029	11-07-84	JP-A- 59110662	26-06-84
		US-A- 4663435	05-05-87
FR-A-2320109	04-03-77	CH-A- 626328	13-11-81
		DE-A- 2635558	17-02-77
		GB-A- 1502573	01-03-78
		JP-A- 52019659	15-02-77
		NL-A- 7607987	10-02-77
		SE-B- 419440	03-08-81
		SE-A- 7608300	09-02-77
		US-A- 4161521	17-07-79